Enhanced glutamine uptake influence composition of immune cells infiltrates in breast cancer

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Running title: Glutamine transporters derives immune cells infiltrates in breast cancer.

Key words: SLC1A5, SLC7A5, SLC3A2, breast cancer, immune cells infiltrates.

Abstract

Background: Cancer cells must alter their metabolism to support proliferation. Immune evasion also plays a role in supporting tumour progression. This study aimed to find whether enhanced glutamine uptake in breast cancer (BC) can derive the existence of specific immune cells subtypes, including the subsequent impact on patient outcome.

Method: SLC1A5, SLC7A5, SLC3A2 and immune cell markers; CD3, CD8, FOXP3, CD20 and CD68, in addition to PD1 and PDL1 were assessed using immunohistochemistry on TMAs constructed from a large BC cohort (n=803). Patients were stratified based on SLC protein expression into accredited clusters and correlated with immune cell infiltrates and patient outcome. The effect of transient siRNA knockdown of SLC7A5 and SLC1A5 on PDL1 expression was evaluated in MDA-MB-231 cells.

Results: High SLCs were significantly associated with PDL1 and PD1+, FOXP3+, CD68+ and CD20+ cells ($p<0.001$). Triple Negative (TN), HER2+ and luminal B tumours showed variable associations between SLCs and immune cell types ($p\leq 0.04$). The expression of SLCs and PDL1, PD1+, FOXP3+ and CD68+ cells was associated with poor patient outcome ($p<0.001$). Knockdown of SLC7A5 significantly reduced PDL1 expression.

Conclusion: This study provides data that altered glutamine pathways in BC appears to play a role in deriving specific subtypes of immune cell infiltrates, which either support or counteract its progression.

Background

Altered metabolic pathways are readily accepted as part of the revised hallmarks of cancer where cancer cells adapt their metabolism in order to resist the unfavourable, nutrient-deprived conditions and to respond to the increased energy demands required by their unremitting proliferation (1). Many cancer cells are highly reliant on amino acids for their growth, not only because they are precursors for nucleotide and protein synthesis, but also because they activate mammalian target of rapamycin complex1 (mTORC1) through nutrient signalling pathways which in turn regulates protein translation and cell growth (2, 3).

Solute carrier family 1 member 5 (SLC1A5) and solute carrier family 7 member 5 (SLC7A5) are two key amino acid transporters which have been attracting attention due to their role in supporting tumour metabolism. Primarily, SLC1A5 maintains the sodium−coupled influx of glutamine, whereas SLC7A5 mediates the efflux of this amino acid in exchange with the influx of leucine, an essential amino acid and potent activator of mTORC1 (4, 5). SLC7A5 requires a covalent association with the heavy chain of SLC3A2, for its functional expression in plasma membrane (6). We have previously described the potential utility of these transporters as prognostic factors in certain BC subtypes (7, 8). Further, we have recently stratified BC patients into three accredited clusters based on the protein expression of these three solute carriers (9).

The role of the tumour microenvironment (TME) is well known with respect to disease development and progression. One of the important components of the TME is immune cells, including the regulatory T-cells (Treg) and tumour associated macrophages (TAM), which gain pro-tumoural functions stimulating tumour growth, progression, invasion, and metastasis. Conversely, other immune cells such as CD8+ and CD20+ lymphocytes are responsible for anti-tumoural responses by activating host defence mechanisms preventing

immune evasion (10, 11). Immune evasion is a strategy used by tumours to evade a host's immune response in an attempt to maximize their probability to continue surviving and growing. Tumour immune evasion include several mechanisms such as progressive formation of an immune suppressive environment within the tumour and the selection of tumour variants resistant to immune effectors (immunoediting) (12).

Composition of the inflammatory cell infiltrates in BC also correlates with clinical outcome, where an abundant infiltration of pro-tumorigenic cells is associated with poor outcome while TME enriched in cells with anti-tumorigenic functions have a favourable effect on patient survival (13-15). Programmed Cell Death 1 (PD1) and its activating ligand, Programmed Death Ligand 1 (PDL1) act in attenuating the anti-cancer immune response and promoting T-regulatory cell development and function. Indeed, PDL1 is expressed by tumour cells of several cancer types, with evidence of an association with aggressive tumour behaviour and poor prognosis (16-20).

Previous studies show that the cellular contents of the TME change in parallel with tumour growth/progression and the accompanied alterations in glucose metabolism in cancer cells is tightly linked to the composition of the surrounding immune cells (21, 22). We therefore hypothesise that the reprogramming of glutamine metabolism will have an impact on the structure of the immune cells. This study aimed to determine whether over-expression of the key glutamine solute carriers can derive the existence of specific subtypes of immune cells, in addition to their supportive role in estimating the clinical outcome.

MATERIAL AND METHODS

Patient Cohort

This study evaluated a well-characterised cohort of early stage, primary operable, invasive BC patients aged ≤ 70 years. Patients (n=803) presented at Nottingham City Hospital from 1989 to 1998. Patient management was uniform and based on tumour characteristics by Nottingham Prognostic Index (NPI) and hormone receptor status. Clinical history, tumour characteristics, information on therapy and outcomes are prospectively maintained. Outcome data included development and time to distant metastasis (DM) and breast cancer specific survival (BCSS), defined as the time (in months) from the date of primary surgical treatment to the time of death from BC. The clinicopathological parameters for the BC series are summarised in (Supplementary table 1).

Tissue microarrays (TMAs) and Immunohistochemistry

TMAs consisting of 0.6mm tumour tissue cores were arrayed and immunohistochemically profiled for SLC1A5, SLC7A5, SLC3A2, PD1, PDL1, CD3, CD8, CD68, CD20 and FOXP3, as previously described (23) (9) (13) (24).

TMA sections stained with SLC1A5, SLC7A5, SLC3A2, PDL1 and PD1 were scanned using high-resolution digital images (NanoZoomer; Hamamatsu Photonics, Welwyn Garden City, UK), at x20 magnification. Evaluation of staining was based on a semi-quantitative assessment using a modified histochemical score (H-score) (25) as previously described (9). Clustering analysis of SLC1A5, SLC7A5 and SLC3A2 protein expression was previously performed using two algorithms, partitioning around medoids (PAM) and K-means, to stratify tumours into the optimal number of clusters based on their H-score. The three clusters were characterised as follows: Low SLCs (SLC1A5-/SLC7A5-/SLC3A2-), High SLC1A5 (SLC1A5+/SLC7A5-/SLC3A2-) and High SLCs (SLC1A5+/SLC7A5+/SLC3A2+) (9).

Immunohistochemical detection of a panel of lymphocyte markers including pan T-cell CD3, cytotoxic T-cell CD8, T-reg FOXP3, B-cell CD20 and Histiocytic cell marker; CD68 was previously determined and the total number of each immune cell type was counted in each tumour core using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) as described (13-15). BC molecular subtypes were defined, based on tumour IHC profile and the Elston-Ellis (26) mitotic score, as: ER+/HER2- Low Proliferation (mitotic score 1), ER+/HER2- High Proliferation (mitotic score 2 and 3), HER2-positive class: HER2+ regardless of ER status, Triple Negative (TN): ER-, PgR- and HER2- (27).

siRNA transfection of SLC7A5 and SLC1A5

The TN cell line, MDA-MB-231, was obtained from American Type Culture Collection; Rockville, MD, USA and cultured in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK). Mycoplasma testing was carried out on a routine basis using the MycoAlert Detection kit (R&D Systems). $5x10^4$ cells were seeded per well in a 24-well plate and transfected using the reverse transfection method with 25 pmol and 100 pmol siRNA (ThermoFisher Scientific), for SLC7A5 and SLC1A5 respectively, and lipofectamine (RNAiMAX) according to the manufacture's protocol.

siRNA anti-sense sequences were as follows: 5'-UUGGGAUCUAGAUUGGACAca-3' (for SLC7A5) and 5'-AAAGAGUAAACCCACAUCCtc-3' (for SLC1A5). Untransfected cells were carried out alongside the experiment as controls. SLC7A5 and SLC1A5 expression of transfected cells was performed in duplicate and determined by Western blotting analysis (Supplementary figure 2A-B).

Statistical analysis

Statistical analysis was performed using SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA). The Chi-square test was performed for inter-relationships between categorical variables. Differences between two groups of normalised data were assessed using t-test. Survival curves were analysed by Kaplan-Meier with Log Rank test. This was performed with BC specific death; those who died of other causes, alive and lost to follow-up were censored. P-values were adjusted using Bonferroni correction for multiple testing. A p-value <0.05 was considered significant. The study endpoints were 10-year breast cancer specific survival (BCSS) or distant metastasis free survival (DMFS).

This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer' and the North West – Greater Manchester Central Research Ethics Committee under the title 'Nottingham Health Science Biobank (NHSB)' reference number 15/NW/0685.

Results

Expression of SLC1A5, SLC7A5, SLC3A2 and immune cell markers in BC

Expression of the three solute carriers was predominantly in the membrane of the invasive BC cells, with intensity levels varying from absent to high. High expression of the solute carriers was also observed in lymphocytic infiltrates, which were in the stroma adjacent to the tumour cells (Figure 1A-1C). Immunohistochemical expression of solute carriers and immune cell markers in invasive BC cores are illustrated in Supplementary figure 1.

Association of SLCs with immune cell infiltrates

Tumour infiltrating FOXP3+ lymphocytes and CD68+ macrophages were both predominant in tumours with High SLCs and to a lesser extent in those tumours with High SLC1A5 expression (Table 1, $p<0.0001$). CD20+ lymphocytes were mainly observed in tumours with High SLCs expression (Table 1, $p<0.0001$). While PDL1 was highly expressed in tumours with High SLCs and High SLC1A5 expression, PD1+ cells were mainly expressed with tumours with High SLCs expression (Table 1, $p<0.0001$). In contrast, there was no significant association between the SLCs clusters and CD3+ or CD8+ T lymphocytes (Table 1, $p=0.84$ and $p=0.24$) respectively.

Association between SLCs clusters and immune cells infiltrates varied among BC molecular subtypes

CD68+ cells were significantly associated with High SLCs expression in both ER+ high proliferative/luminal B and TN subtypes (Table 2 and 3, $p=0.02$ and $p=0.03$) respectively. In contrast, FOXP3+ cells were only associated within TN tumours showing High SLCs expression (Table 3, $p=0.02$). CD20+ cells were only observed in ER+ high proliferative/

luminal B tumours with High SLCs (Table 2, p=0.04). PD1 and PDL1 were also significantly expressed in HER2+ tumours with High SLCs expression (Table 3, p=0.03 and p=0.04) respectively. However, within TN tumours, only PDL1 expression was associated with High SLCs expression (Table 3, p=0.04). There were no significant associations between the SLCs clusters and immune cells infiltrates in ER+ low proliferative/luminal A tumours (Table 2).

The co-occurrence of SLCs and immune cells infiltrates correlates with patient outcome

Variable associations with patient outcome were observed when investigating the co-occurrence of the SLCs clusters with the immune cell markers. The co-existence of high SLCs with FOXP3+ T lymphocytes was predictive of a shorter BCSS (Figure 2A, $p<0.001$). Similar associations were observed when CD68+ macrophages, PD1+ cells and PDL1 expression, were considered (Figure 2B-D, $p<0.001$). However, patients with tumours showing both high SLCs and CD20+ lymphocytes, showed better BCSS (Figure 2E, $p<0.001$).

There was a comparable observation regarding the association of SLCs and immune cell markers with DMFS, where high SLCs expression accompanied by the presence of either FOXP3+, CD68+, PD1+ cells or PDL1 expression showed significantly shorter DMFS (Supplementary figure 3A-D, all $p<0.001$). In contrast, the presence of CD20+ lymphocytes and High SLCs conferred a longer DMFS (Supplementary figure 3E, p=0.002).

SLC7A5 plays a role in PDL1 expression in TNBC

Functional analysis was carried out using the TNBC cell line, MDA-MB-231, due to the high expression of PDL1 together with SLC7A5 and/or SLC1A5 expression and also based on the significant associations found between SLCs and immune cell markers, including PDL1, within the TN BC subtype (Figure 3B-C).

siRNA knockdown of SLC1A5 or SLC7A5 in MDA-MB-231 reduced the protein expression of PDL1. However, this observation was only significant upon targeting SLC7A5 (Figure 3D, $p=0.01$) but not SLC1A5 (Figure 3E, $p=0.13$). Significant reduction of PDL1 protein expression was also observed in cells transfected with both SLC1A5 and SLC7A5 siRNAs. (Figure 3F, $p=0.02$).

Discussion

Breast cancer is a heterogeneous disease with various subtypes (28) that are different in terms of morphology, molecular profiles, response to therapy and clinical behaviour. Breast cancer also shows heterogeneity in metabolic reprogramming, where highly proliferative tumours are distinguished based on their metabolic signatures (29-31).

Cancer cells undergo metabolic changes in order to satisfy the demands of necessary energy and cellular building blocks. One of the most prominent is the increase in glutamine consumption which is reflected by the up-regulation of the key glutamine transporters (SLC1A5 and the SLC7A5-SLC3A2 dimeric complex) at the surface of the tumour cells. We have recently demonstrated that the combined expression of the three solute carriers (SLC1A5, SLC7A5 and SLC3A2) is associated with poor prognosis and short BCSS, particularly in the highly proliferative BC subtypes (9).

Besides metabolic reprogramming, immune evasion is also considered as an emerging hallmark of cancer (1). The role of immune cells in tumour evasion is increasingly barbed, as many tumours not only escape recognition by the adaptive immune response but also sometimes cooperate with the pro-tumourigenic immune cells to become invasive and more aggressive. Furthermore, there is a link between the two mentioned hallmarks, as changes in the tumour cell metabolism can influence the component and function of the inflammatory infiltrates (21, 32). This study showed that altered glutamine metabolism, which was detected by the over-expression of the key glutamine transporters (SLC1A5, SLC7A5 and SLC3A2) was significantly associated with the existence of specific subtypes of immune cells, namely CD68+ macrophages, FOXP3+ Regulatory T Cells (Tregs), CD20+ B lymphocytes and PD1+ Lymphocytes along with its tumour-expressing ligand (PDL1). However, no association was observed between the SLCs and CD3+ or CD8+ T lymphocytes.

Our previous study on the same BC cohort, showed that the main component of the inflammatory infiltrates is the pan T lymphocyte population (CD3+ cells) along with CD8+ cells being more frequent than FOXP3+ cells. The CD68+ macrophages were more frequent while CD20+ B lymphocyte were the least (33). In this study, however, we observed that changes in the metabolic activity of the cancer cells, which is reflected by an increase in glutamine transport, derive specific components of immune cells which was restricted to CD68+, FOXP3+, CD20+ along with PD1+ cells. This indicates that in these circumstances, the Antigen Presenting Cells (APC), CD68+ cells, are recognised only by specific subpopulations of T and B-lymphocytes.

When different BC subtypes were examined, a significant association was observed in ER+ highly proliferative/luminal B, TN and HER2+ tumours, but not the ER+ low proliferative/ luminal A subtype. Both luminal B and TN tumours showed associations with CD68+ macrophages. These two subtypes are aggressive, highly proliferative and exhibit high metabolic activity. Consequently, aggressive cancer cells secrete high levels of reactive oxygen species (ROS) in their microenvironment (34). The latter can cause a state of pseudohypoxia in the adjacent stromal compartment with concomitant up-regulation of Hypoxia Inducible Factor 1α (HIF1α), known to induce the pro-tumourigenic CD+68 macrophages (35, 36). The same scenario can be applied when amino acids, particularly leucine, activate mTORC1 which upregulates HIF1α (37). Previous studies have shown that PD1/PDL1 are mainly expressed in HER2+ and TN subtypes (20, 38, 39). This study further shows that the expression of PD1 and/or PDL1 is mainly associated with high SLCs expression but restricted to HER2+ and TN tumours.

In this study, we observed high expression of the solute carriers in the stromal lymphocytes. This is expected as glutamine transporters are not only necessary for cancer cells, they are

also important for optimal lymphocyte proliferation and differentiation (40-43). Additionally, macrophages may require glutamine as it is the main precursor for arginine (44). The latter can be catalysed by Arginase 1 to support cell proliferation and tissue remodelling (45).

Indeed, the up-regulation of glutamine transporters in the cancer cells and their neighbouring immune cells, might indicate that both cell types are substantially comparable in their requirements of amino acids, which can be obtained from the TME, to support their survival and proliferation. Furthermore, TME might be a source of stromal glutamine, as it has been found that the metabolic stress in TME triggers genomic instability, which subsequently acquire the non-malignant cancer-associated fibroblasts (CAF) a catabolic phenotype with enhanced macroautophagy. This catabolic state produces a nutrient-rich environment, with increased amounts of pyruvate, lactate, ketone bodies and glutamine (46). This phenomenon also substantiates the cancer-stromal symbiosis which subsequently supports cancer growth and progression.

We and others revealed that high expression of glutamine solute carriers is associated with poor patient outcome (7, 8, 47). Similarly, the presence of FOXP3+ and CD68+ cells also correlate with shorter survival (33). However, CD20+ cells tend to be associated with better survival (33). In this study we showed that the co-occurrence of SLCs with FOXP3+ and CD68+ cells can predict shorter survival compared to the presence of one without the other, whereas the combination of the SLCs with CD20+ cells derive better patient outcome.

The association between PD1/PDL1 and survival in BC is controversial (20, 48-50). This study, however, showed that the co-expression of SLCs with PD1 or PDL1 was associated with shorter distant metastasis free survival and breast cancer specific survival, indicating that patients with BC showing an increase in their amino acid metabolic activity which might influence poor outcome in PD1/PDL1+ tumours.

We observed that targeting SLC7A5 by transient siRNA significantly reduced the expression of PDL1 in TN cells. This can be attributed to the role played by this protein in activating the mTORC1 pathway, through importing essential amino acids, such as leucine. This might take place in parallel with the activation of mTORC1 through the AKT-mTOR signalling pathway, which is previously identified as a tight regulator of PDL1 expression in several cancers, including TNBC (38, 51, 52).

Although clinical trials with monoclonal antibodies targeting PD1/PDL1 interaction have shown promising results, with durable responses, in several human cancers (53-55), not all patients respond to this targeted therapy. Therefore, it is critical to find effective approaches that could allow personalisation of treatment of PD1/PDL1+ tumours. This study not only provides clinical evidence that SLCs in BC could aid the personalisation of anti-PD1/PDL1 inhibition therapies, it also emphasises that targeting the amino acid transporter, SLC7A5, along with the anti-PDL1 immunotherapy could be considered as a novel approach to synergistically enhance the therapeutic effect.

Conclusion

This study revealed that there are associations between the two cancer hallmarks, metabolic reprogramming and immune evasion. Altered glutamine pathways in cancer cells can derive specific subtypes of inflammatory infiltrates, which acts either with or against the aggressiveness and progression of the BC cells. Targeting both SLC7A5 and PD1/PDL1 can be a new approach which will counteract the highly proliferative and aggressive BC subtypes.

Declarations

Ethics approval and consent to participate: This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer' and the North West – Greater Manchester Central Research Ethics Committee under the title 'Nottingham Health Science Biobank (NHSB)' reference number 15/NW/0685.

Consent to publish: Not applicable.

Data availability: The datasets generated during the current study are available from the Corresponding Author on reasonable request.

Conflict of interests: The authors declare no conflict of interests.

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Authors' contributions: RE contributed to writing, IHC staining, scoring, data analysis and interpretation; MLC contributed to writing and reviewing the manuscript; MA and LA contributed to analysis and reviewing the manuscript; IOE and EAR contributed to writing and reviewing the manuscript; ARG contributed to study design, data analysis and interpretation, writing and reviewing the manuscript.

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Table 1: Association of SLCs with different subtypes of immune cell markers					
Immune cell marker	**Low SLCs**	**High SLC1A5**	**High SLCs**	**χ2**	**Adjusted**
	n (%)	**n (%)**	**n (%)**	**(p-value)**	**p-value**
CD3					
Negative	36 (56.3)	21 (32.8)	7 (10.9)	1.7	
Positive	268 (54.1)	142 (28.7)	85 (17.2)	(0.42)	0.84
CD8					
Negative	71 (57.7)	35 (28.5)	17 (13.8)	4.9	
Positive	236 (48.9)	140 (29.0)	107 (22.2)	(0.08)	0.24
FOXP3					
Negative	148 (63.8)	67 (28.9)	17 (7.3)	37.3	
Positive	188 (44.9)	122 (29.1)	109 (26.0)	(7.9x10-9)	**<0.0001**
CD68					
Negative	139 (67.1)	47 (22.7)	21 (10.1)	35	
Positive	165 (42.7)	124 (32.1)	97 (25.1)	(2.5x10-8)	**<0.0001**
CD20					
Negative	255 (55.4)	134 (29.1)	71 (15.4)	17.1	
Positive	81 (44.5)	47 (25.8)	54 (29.7)	(0.0001)	**0.0004**
PD1					
Negative	203 (52.1)	111 (28.5)	76 (19.5)	42.5	
Positive	134 (36.1)	85 (22.9)	152 (41.0)	(6.0x10-10)	**<0.0001**
PDL1					
Negative	81 (67.5)	28 (23.3)	11 (9.2)	16.8	
Positive	287 (48.2)	178 (29.9)	130 (21.8)	(0.0002)	**0.0004**

Table 2: Association of SLCs with immune cell markers in ER+ low proliferative/luminal A and ER+ high proliferative/luminal B BC subtype									
	ER+ low proliferative/luminal A					ER+ high proliferative/luminal B			
Immune cell marker	**Low SLCs**	**High SLC1A5**	**High SLCs**	**χ2**	**Adjusted**	**Low SLCs**	**High SLC1A5**	**High SLCs**	**χ2**
	n (%)	**n (%)**	**n (%)**	**(p-value)**	**p-value**	**n (%)**	**n (%)**	**n (%)**	**(p-value)**
CD3									
Negative	10 (66.7)	4 (26.7)	1 (6.7)	1.41	3.92	10 (41.7)	12 (50.0)	2 (8.3)	1.31
Positive	95 (79.8)	20 (16.8)	4 (3.4)	(0.49)		93 (52.5)	67 (37.9)	17 (9.6)	(0.51)
CD8									
Negative	22 (75.9)	5 (17.2)	2 (6.9)	0.22	6.23	20 (47.6)	16 (38.1)	6 (14.3)	0.56
Positive	82 (78.8)	17 (16.3)	5 (4.8)	(0.89)		84 (50.6)	65 (39.2)	17 (10.2)	(0.75)
FOXP3									
Negative	61 (80.3)	12 (15.8)	3 (3.9)	0.94	3.72	47 (55.3)	34 (40.0)	4 (4.7)	3.76
Positive	50 (73.5)	14 (20.6)	4 (5.9)	(0.62)		69 (51.1)	49 (36.3)	17 (12.6)	(0.15)
CD68									
Negative	55 (85.9)	7 (10.9)	2 (3.1)	4.75	0.45	50 (66.7)	21 (28.0)	4 (5.3)	10.6
Positive	47 (70.1)	15 (22.4)	5 (7.5)	(0.09)		56 (43.8)	54 (42.2)	18 (14.1)	(0.005)
CD20									
Negative	92 (79.3)	19 (16.4)	5 (4.3)	0.56	3.00	83 (50.3)	69 (41.8)	13 (7.9)	8.03
Positive	20 (74.1)	5 (18.5)	2 (7.4)	(0.75)		32 (60.4)	12 (22.6)	9 (17.0)	(0.01)
PD1									
Negative	85 (79.4)	19 (17.8)	3 (2.8)	3.01	0.66	64 (50.8)	53 (42.1)	9 (7.1)	6.02
Positive	42 (71.2)	12 (20.3)	5 (8.5)	(0.22)		54 (50.5)	35 (32.7)	18 (16.8)	(0.04)
PDL1									
Negative	24 (82.8)	4 (13.8)	1 (3.4)	0.6	1.46	19 (70.4)	6 (22.2)	2 (7.4)	3.64
Positive	99 (76.2)	24 (18.5)	7 (5.4)	(0.73)		109 (0.9)	80 (37.4)	25 (11.7)	(0.15)

Table 3: Association of SLCs with immune cell markers in HER2+ and Triple Negative BC subtypes									
	HER2+					Triple Negative			
Immune cell marker	**Low SLCs**	**High SLC1A5**	**High SLCs**	**χ2**	**Adjusted**	**Low SLCs**	**High SLC1A5**	**High SLCs**	**χ2**
	n (%)	**n (%)**	**n (%)**	**(p-value)**	**p-value**	**n (%)**	**n (%)**	**n (%)**	**(p-value)**
CD3									
Negative	3 (75.0)	1 (25.0)	0 (0.0)	3.02	1.76	3 (30.0)	4 (40.0)	3 (30.0)	4.68
Positive	22 (33.8)	26 (40.0)	17 (26.2)	(0.22)		16 (25.4)	9 (14.3)	38 (60.3)	(0.09)
CD8									
Negative	5 (62.5)	3 (37.5)	0 (0.0)	4.37	0.77	6 (30.0)	7 (35.0)	7 (35.0)	7.72
Positive	23 (31.1)	30 (40.5)	21 (28.4)	(0.11)		18 (21.2)	11 (12.9)	56 (65.9)	(0.02)
FOXP3									
Negative	6 (46.2)	6 (46.2)	1 (7.7)	2.47	1.74	9 (42.9)	6 (28.6)	6 (28.6)	9.86
Positive	22 (30.6)	31 (43.1)	19 (26.4)	(0.29)		18 (19.8)	13 (14.3)	60 (65.9)	(0.007)
CD68									
Negative	4 (26.7)	4 (26.7)	7 (46.7)	5.18	0.35	3 (20.0)	7 (46.7)	5 (33.3)	10.1
Positive	24 (35.3)	31 (45.6)	13 (19.1)	(0.07)		18 (21.4)	11 (13.1)	55 (65.5)	(0.007)
CD20									
Negative	20 (42.6)	17 (36.2)	10 (21.3)	3.05	0.84	13 (21.7)	13 (21.7)	34 (56.7)	1.55
Positive	9 (24.3)	18 (48.6)	10 (27.0)	(0.21)		12 (25.0)	6 (12.5)	30 (62.5)	(0.46)
PD1									
Negative	14 (37.8)	20 (54.1)	3 (8.1)	8.57	0.03	11 (29.7)	7 (18.9)	19 (51.4)	1.35
Positive	16 (29.1)	20 (36.4)	19 (34.5)	(0.01)		19 (20.4)	18 (19.4)	56 (60.2)	(0.50)
PDL1									
Negative	8 (47.1)	9 (52.9)	0 (0.0)	7.4	0.04	7 (50.0)	3 (21.4)	4 (28.6)	7.48
Positive	19 (26.0)	32 (43.8)	22 (30.1)	(0.02)		21 (19.4)	20 (18.5)	67 (62.0)	(0.02)

Adjusted p-value
0.72
0.14
0.02
0.03
1.84
1.5
0.04

Adjusted	
p-value	
4.08	
5.25	
0.9	
0.02	
0.04	
0.12	
0.3	

Figure legends

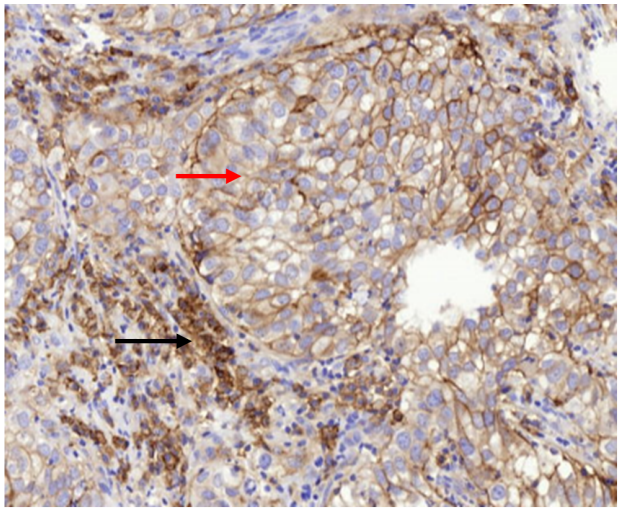
Figure 1. The expression of solute carriers was predominant in the cell membrane of the breast cancer cells (Red arrows) and the adjacent immune cells infiltrates (Black arrows). A)

Figure 2. Breast cancer specific survival in SLCs and immune markers co-expression. A) SLCs and FOXP3. B) SLCs and CD68. C) SLCs and PD1. D) SLCs and PDL1. E) SLCs and CD20.

Figure 3. PDL1 protein expression in western blotting. A) Western blot analysis of PDL1 protein expression in MDA-MB-231 cells transfected with SLC7A5 and/or SLC1A5 SiRNA. Western blot results in different BC cell lysates for B) SLC7A5 and C) SLC1A5. The bar graph summarises the expression levels of PDL1 protein, using β-actin as normalised control, upon D) SLC7A5 SiRNA transfection. E) SLC1A5 SiRNA transfection. F) SLC7A5 and SLC1A5 SiRNA transfection. Data represent the mean and error bars of three independent experiments.

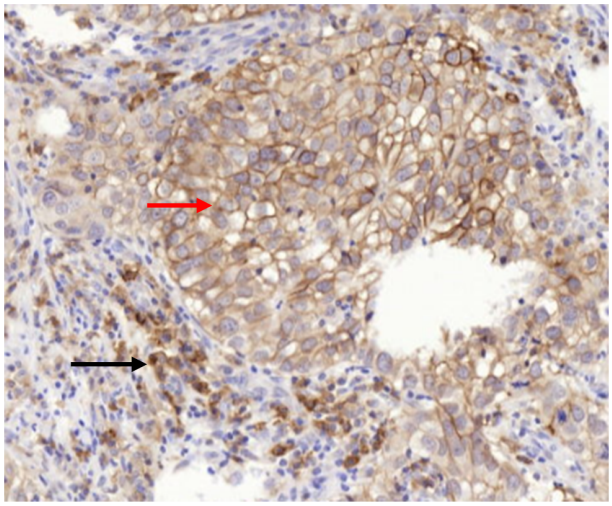
a

SLC1A5



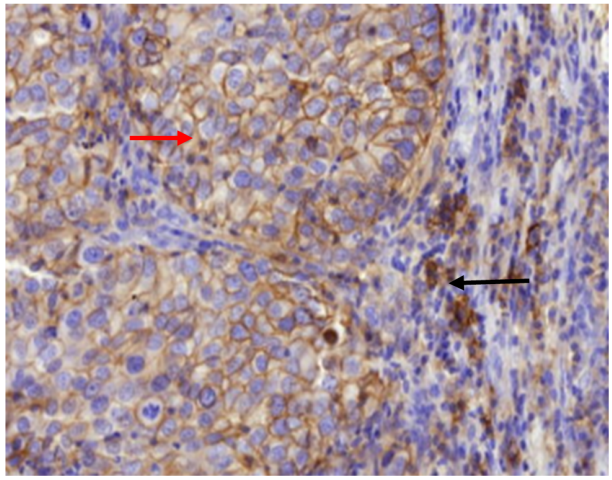
b

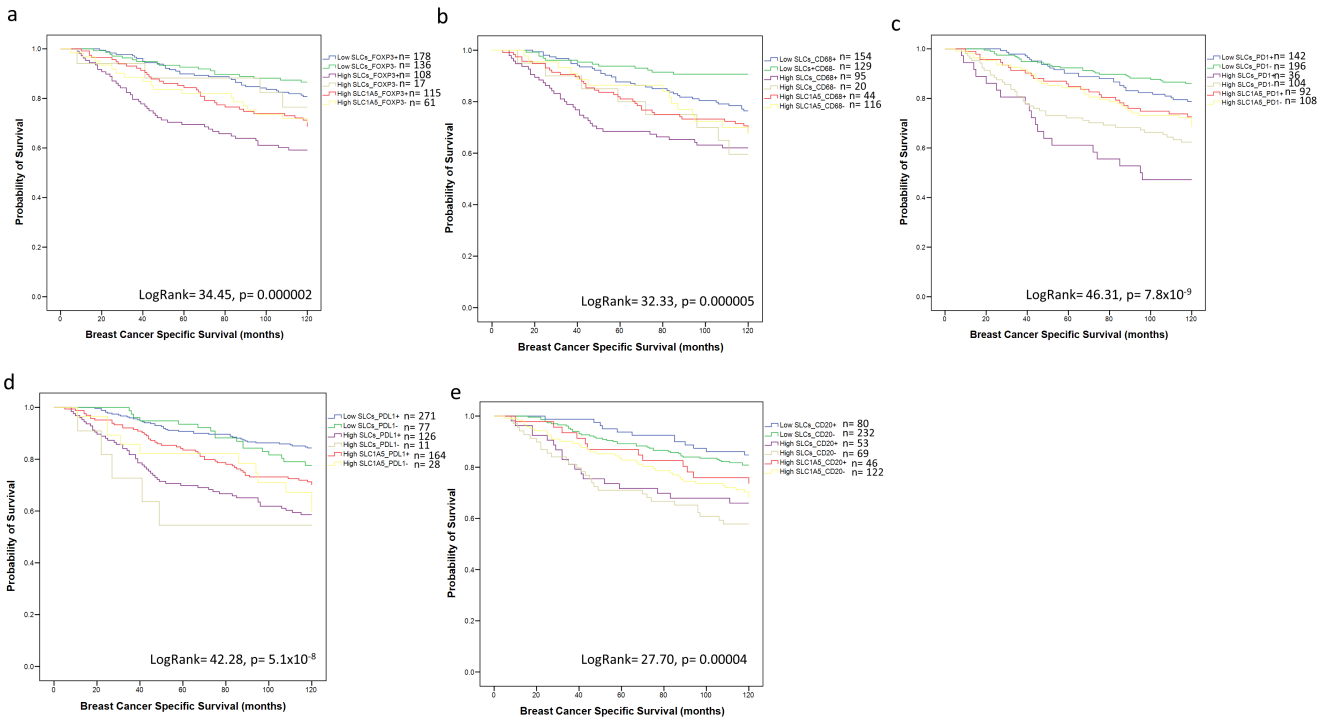
SLC7A5

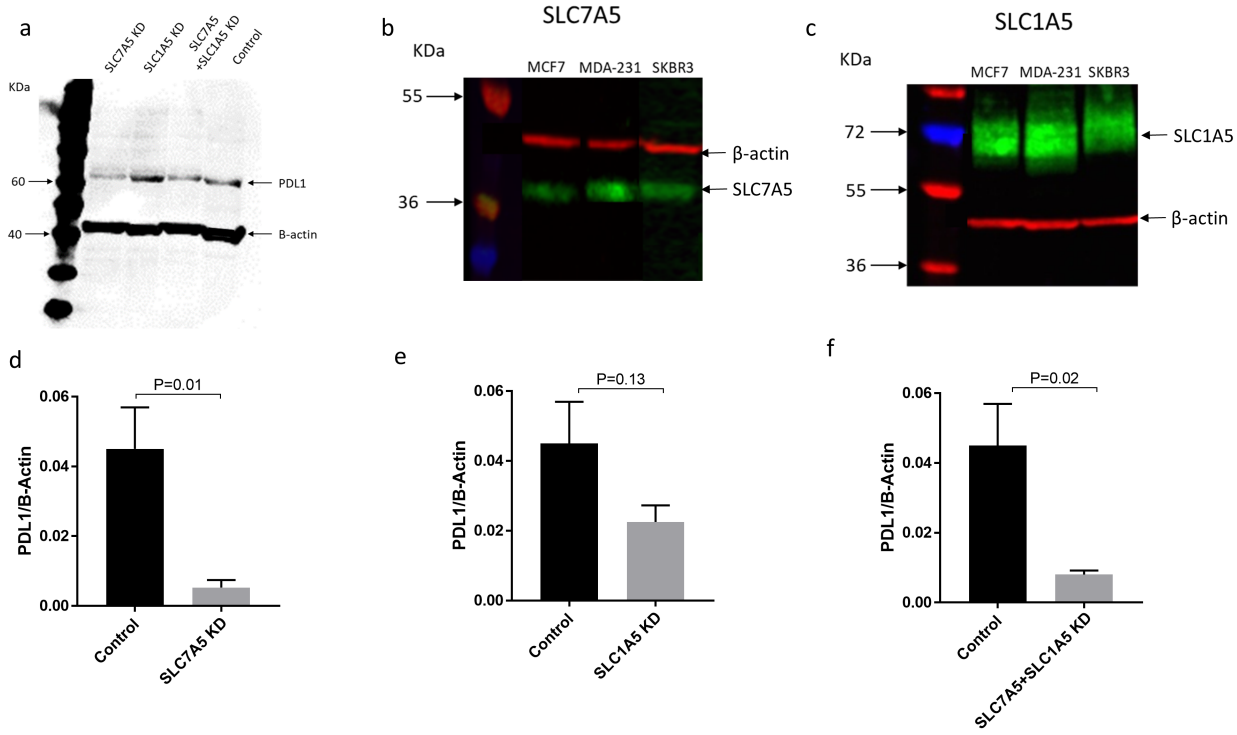


C

SLC3A2



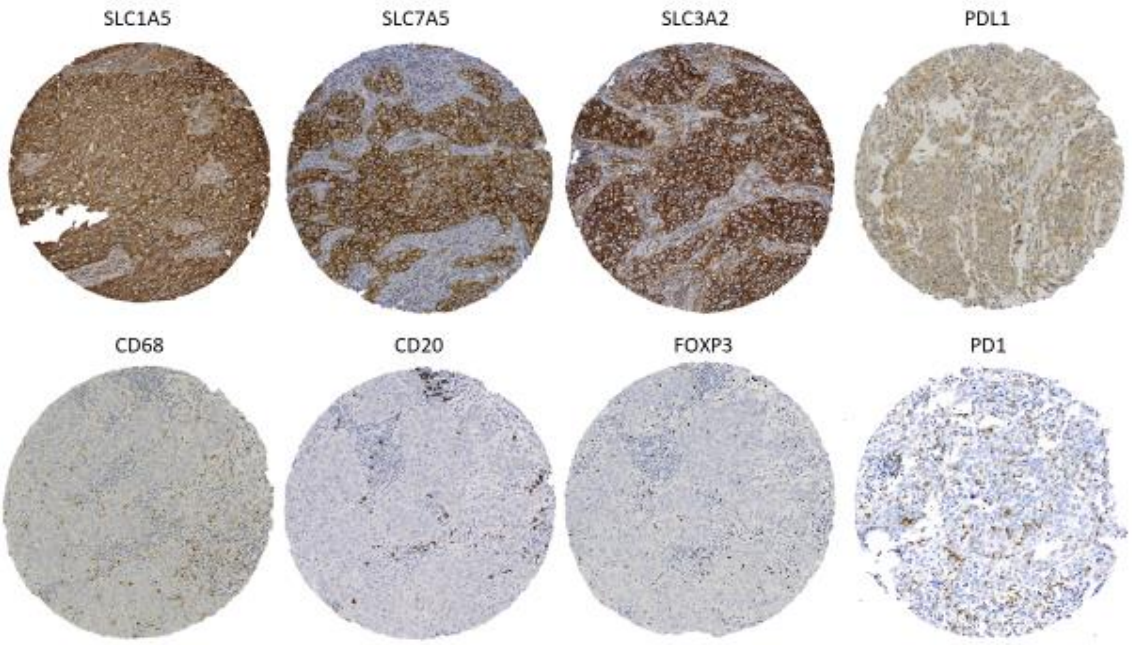

a
Low SLCs_FOXP3+ n= 178
Low SLCs_FOXP3- n= 136
High SLCs_FOXP3+ n= 108
High SLCs_FOXP3- n= 17
High SLC1A5_FOXP3+ n= 115
High SLC1A5_FOXP3- n= 61
Probability of Survival
LogRank= 34.45, p= 0.000002
Breast Cancer Specific Survival (months)
b
Low SLCs_CD68+ n= 154
Low SLCs+CD68- n= 129
High SLCs_CD68+ n= 95
High SLCs_CD68- n= 20
High SLC1A5_CD68+ n= 44
High SLC1A5_CD68- n= 116
Probability of Survival
LogRank= 32.33, p= 0.000005
Breast Cancer Specific Survival (months)
c
Low SLCs_PD1+ n= 142
Low SLCs_PD1- n= 196
High SLCs_PD1+ n= 36
High SLCs_PD1- n= 104
High SLC1A5_PD1+ n= 92
High SLC1A5_PD1- n= 108
Probability of Survival
LogRank= 46.31, p= 7.8x10-9
Breast Cancer Specific Survival (months)
d
Low SLCs_PDL1+ n= 271
Low SLCs_PDL1- n= 77
High SLCs_PDL1+ n= 126
High SLCs_PDL1- n= 11
High SLC1A5_PDL1+ n= 164
High SLC1A5_PDL1- n= 28
Probability of Survival
LogRank= 42.28, p= 5.1x10-8
Breast Cancer Specific Survival (months)
e
Low SLCs_CD20+ n= 80
Low SLCs_CD20- n= 232
High SLCs_CD20+ n= 53
High SLCs_CD20- n= 69
High SLC1A5_CD20+ n= 46
High SLC1A5_CD20- n= 122
Probability of Survival
LogRank= 27.70, p= 0.00004
Breast Cancer Specific Survival (months)


a
SLC7A5 KD
SLC1A5 KD
SLC7A5 +SLC1A5 KD
Control
KDa
60
40
PDL1
B-actin
b
SLC7A5
KDa
MCF7 MDA-231 SKBR3
55
36
β-actin
SLC7A5
c
SLC1A5
KDa
MCF7 MDA-231 SKBR3
72
55
36
SLC1A5
β-actin
d
P=0.01
PDL1/B-Actin
0.06
0.04
0.02
0.00
Control
SLC7A5 KD
e
P=0.13
PDL1/B-Actin
0.06
0.04
0.02
0.00
Control
SLC1A5 KD
f
P=0.02
PDL1/B-Actin
0.06
0.04
0.02
0.00
Control
SLC7A5+SLC1A5 KD

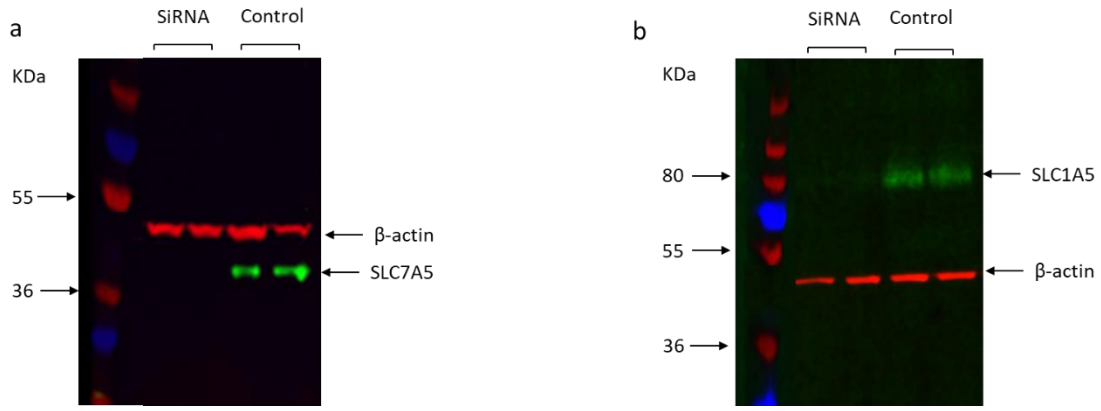
Supplementary table 1: Clinicopathological parameters of the Nottingham BC series

Parameters	Nottingham TMA series n (%)
Tumour size	
≤ 2cm	658 (51.6)
> 2cm	616 (48.4)
Grade	
1	207 (16.3)
2	415 (32.7)
3	649 (51.1)
Tumour type	
Ductal (including mixed)	1052 (82.6)
Lobular	115 (9.0)
Medullary-like	34 (2.7)
Miscellaneous	9 (0.7)
Special type	64 (5.0)
Lymph Node Stage	
1	774 (60.9)
2	396 (31.2)
3	101 (7.9)
Follow-up Status	
Alive	650 (51.0)
Died from Breast Cancer	425 (33.4)
Died from other causes	199 (15.6)
ER	
Negative	325 (25.8)
Positive	934 (74.2)
PgR	
Negative	509 (41.8)
Positive	709 (58.2)
HER2	
Negative	1051 (86.7)

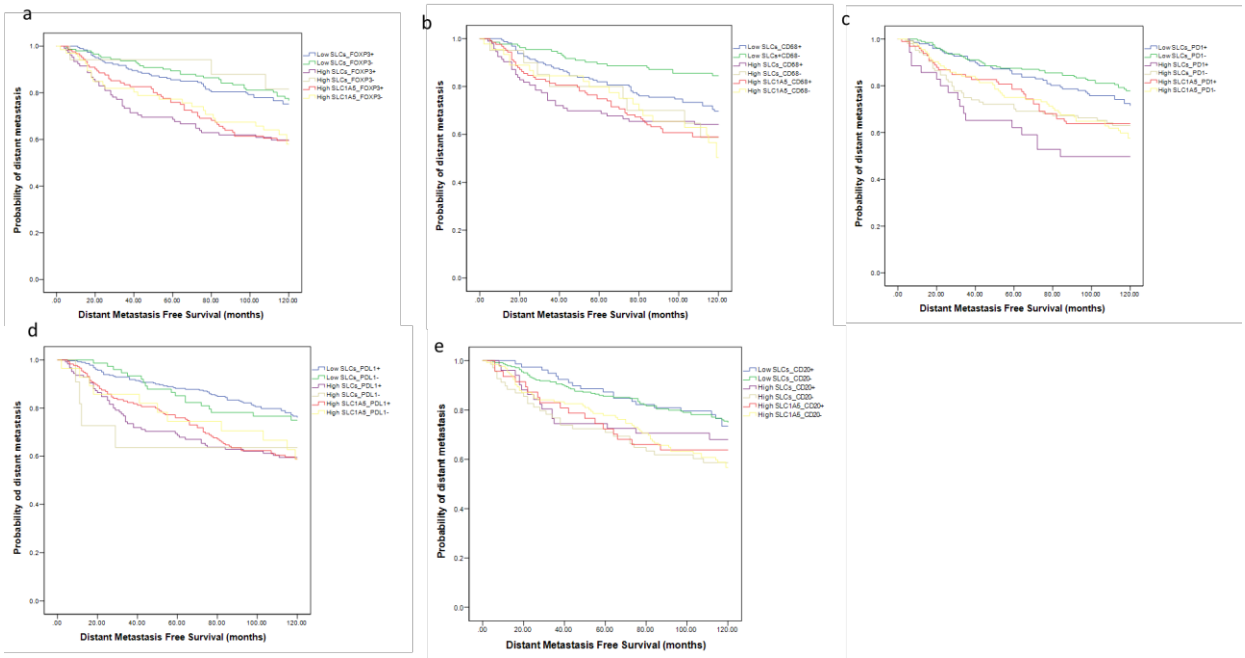
Positive	161 (13.3)



Supplementary figure 1. Immunohistochemical expression of SLC1A5, SLC7A5, SLC3A2 and PDL1 in invasive BC tumour cells (upper panel). Immunohistochemical expression of CD68, CD20, FOXP3 and PD1 in lymphocytic infiltrates of the invasive BC cores (lower panel).



Supplementary figure 2. Knockdown of SLC7A5 and SLC1A5 in MDA-MB-231 cell lines. A) SLC7A5 expression in MDA-MB-231 transfected with SLC7A5 SiRNA and control (un-transfected) cells. B) SLC1A5 expression in MDA-MB-231 transfected with SLC1A5 SiRNA and control (un-transfected) cells.



Supplementary figure 3. Distant metastasis Free Survival in SLCs and immune markers co-expression. A) SLCs-FOXP3. B) SLCs-CD68. C) SLCs-PD1. D) SLCs-PDL1. E) SLCs-CD20.